

Production of biosurfactants using substrates from renewable-resources

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Abstract

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Surface-active compounds commonly used in industries are chemically synthesized. However, biosurfactants have been paid increasing attention to replace the synthetic surfactants owing to their advantages such as biodegradability and low toxicity. Nowadays, the use of biosurfactant has been limited due to the high production cost. Nevertheless, biosurfactants can be produced with high yield by some microorganisms, especially *Pseudomonas* sp. These microorganisms can use the various renewal resources, especially agro-industrial wastes, as the potential carbon sources. This leads to the greater possibility for economical biosurfactant production and reduced pollution caused by those wastes.

Key words : biosurfactant, bioemulsifier, renewable-resources, surface-active compounds

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บทคัดย่อ

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การผลิตสารลดแรงตึงผิวชีวภาพจากวัสดุเศษเหลือ

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สารลดแรงตึงผิว (surface-active compound) ที่ใช้ในอุตสาหกรรมส่วนใหญ่เป็นสารที่สังเคราะห์ขึ้นทางเคมี อย่างไรก็ตามสารลดแรงตึงผิวที่ได้จากสิ่งมีชีวิต (biosurfactant) ได้รับความสนใจมากขึ้น เนื่องจากมีข้อดีคือ สามารถย่อยสลายได้และมีความเป็นพิษต่ำ ส่งผลให้สารลดแรงตึงผิวที่ได้จากสิ่งมีชีวิตเข้ามาแทนที่สารลดแรงตึงผิวที่ได้จากการสังเคราะห์ทางเคมี แต่การใช้สารลดแรงตึงผิวที่ได้จากสิ่งมีชีวิตยังคงจำกัดอยู่ในอุตสาหกรรมบางประเภทเท่านั้น เนื่องจากมีต้นทุนการผลิตสูง อย่างไรก็ตามสารลดแรงตึงผิวจากสิ่งมีชีวิตสามารถผลิตจากเชื้อจุลินทรีย์บางชนิด โดยเฉพาะ *Pseudomonas* sp. ซึ่งให้สารลดแรงตึงผิวที่มีคุณสมบัติดีและผลผลิตสูง รวมทั้งสามารถใช้แหล่งคาร์บอนจากวัสดุเศษเหลือจากการแปรรูปทางการเกษตรหลายชนิด ส่งผลให้การผลิตสารลดแรงตึงผิวจากสิ่งมีชีวิตมีความเป็นไปได้ในทางเศรษฐกิจเพิ่มขึ้น และเป็นการลดมลพิษจากวัสดุเศษเหลือดังกล่าว

ภาควิชาเทคโนโลยีชีวภาพอุตสาหกรรม คณะอุตสาหกรรมเกษตร มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90110

Surfactants and emulsifiers are widely used for industrial, agricultural, food, cosmetic and pharmaceutical applications. Most of the compounds are chemically synthesized. However, it is only in the past few decades that surface-active molecules of microbial origin, referred to as biosurfactants, have gained considerable interest. Biosurfactants have advantages over their chemicals counterparts because they are biodegradable (Zajic *et al.*, 1977), have low toxicity (Poremba *et al.*, 1991), are effective at extreme temperatures or pH values (Cameotra and Makkar, 1998) and show better environmental compatibility (Georgiou *et al.*, 1990). Nevertheless, from an economic standpoint, biosurfactants are not yet competitive with the synthetics. Biosurfactants can only replace synthetic surfactants if the cost of the raw material and the process is minimal. So far, several renewable substrates from various sources, especially from industrial wastes have been intensively studied for microorganism cultivation and surfactant production at an experimental scale.

Olive oil mill effluent (OOME): Olive oil extraction involves an intensive consumption of water and produces large amounts of olive oil mill wastewater, thus causing deleterious environmental effects. OOME is a black liquor and consists of a high content of organic matter (20-60 kg COD/m³),

depending on the olive oil extraction procedure (Marques, 2001). OOME contains toxic substances such as polyphenols (Hamman *et al.*, 1999; Marques, 2001), but also valuable organic substances such as sugars, nitrogen compounds, organic acids and residual oils (Mercade' *et al.*, 1993).

Mercade' *et al.* (1993) found that *Pseudomonas* sp. JAMM could reduce the surface tension in culture medium comprising OOME (100 g/l) and NaNO₃ (2.5 g/l). Besides the ability to reduce initial COD from 24.0 g/l to 13.6 g/l after 72 h of incubation, it also decreased total phenol content by 55%. Surface-active compounds produced from *Pseudomonas* sp. JAMM cultured in OOME medium included rhamnolipids biosurfactant, β , β (2-*o*- α -L-rhamnopyranosiloxi) decanoyl decanoic acid and β , β (2-*o*- α -L-rhamnopyranosil- α -rhamnopyranosiloxi) decanoiloxi decanoic acid. A total conversion yield was estimated to be 14g of rhamnolipids per kg of OOME after 150h of cultivation time.

Animal fat: Animal fat and tallow can be obtained at large quantities from meat processing industries and have been used as a cooking medium for foods. However, these fats have recently lost most of the market share to vegetable oils owing to the health concern. Deshpande and Daniels (1995)

used animal fat for the production of sophorolipid biosurfactant by yeast, *Candida bombicola* (Figure 1). When only fat was provided as a sole carbon source, the growth was poor. The mixture of 10% glucose and 10% fat gave the highest level of growth. Sophorolipid was produced at levels of 97 g/l and 12 g/l without and with pH control, respectively.

Frying oil: Used edible oils and fats are considered as a problematic waste, contributing to the environmental pollution. It is well known that microorganisms are able to grow on vegetable oils or fats and produce new products with potential industrial application such as lipase (Haba *et al.*, 2000a) and biodiesel (Alcantara *et al.*, 2000; Cvengroš and Cvengrošova, 2004).

Haba *et al.* (200b) used olive or sunflower cooking oil as carbon source for biosurfactant production by 36 isolated bacteria. Most of the *Pseudomonas* strains tested showed satisfactory growth when cultivated on either used olive oil or used sunflower oil. However, sunflower oil was not as good a substrate as olive oil, either for cell growth or for biosurfactant production. *Pseudomonas* strains decreased the surface tension of the medium to 34-36 mN/m and the emulsions with kerosene remained stable for three months. Biosurfactants produced from *Pseudomonas* 47T2 NCIB 400044 had the molecular mass of 488 Da and 633 Da and were identified as L- α -rhamnopyranosyl- β -hydroxydecanoyl- β -hydroxydecanoate (rhamnolipids 1) and 2-o- α -L-rhamnopyranosyl- α -L-rhamnopyranosyl-b-hydroxydecanoyl- β -hydroxydecanoate (rhamnolipids 2), respectively (Figure 2). This strain gave a final production of rhamnolipids of 2.7 g/l as rhamnose and a production yield of 0.34 g/g.

Soapstock: Soapstock is a gummy, amber-colored by-product of oilseed processing. It is produced when hexane and other chemicals are used to extract and refine edible oil from the seeds.

Shabtai (1990) reported the production of two extracellular capsular heteropolysaccharides, emulsan and biodispersan by *Acinetobacter calcoaceticus* RAG-1 and *A. calcoaceticus* A2, respectively using soap stock as a carbon source. Emulsan (Figure 3) forms and stabilizes oil-in-water emulsion (Kim *et al.*, 2000), whereas biodispersan disperses the large solid limestone granules, forming micrometer-size water suspension (Rosenberg *et al.*, 1988). Both polysaccharides are synthesized within the cell, exported to their outer surface to form an extracellular cell-associated capsule and released subsequently into the growth medium. After 50 h and 45 h of the fermentation, emulsan and biodispersan at levels of 25g/l and 12 g/l were produced, respectively.

Pseudomonas aeruginosa strain LBI, which was isolated from petroleum contaminated soil, produced surface-active rhamnolipids (RL_{LBI}) by batch cultivation in a mineral salts medium with soapstock as the sole carbon source (Benincasa *et*

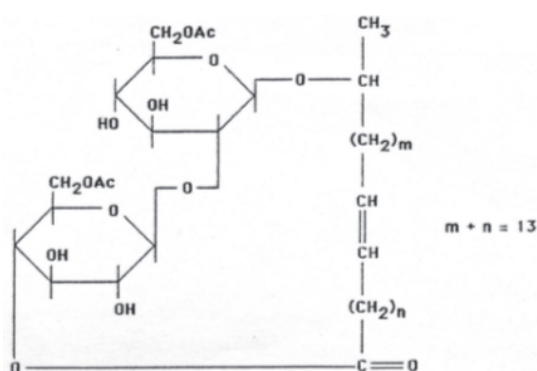


Figure 1. Structure of sophorolipid lactone (Deshpande and Daniels, 1995).

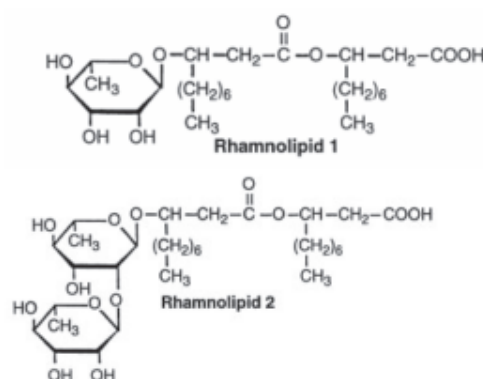


Figure 2. Structure of rhamnolipids from *Pseudomonas* 47T2 NCIB 400044 (Lang and Wullbrandt, 1999).

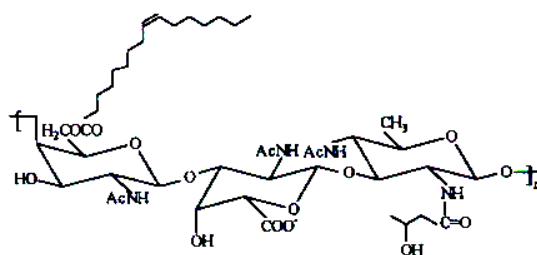


Figure 3. The structure of emulsan-like polymer (Zhang *et al.*, 1997).

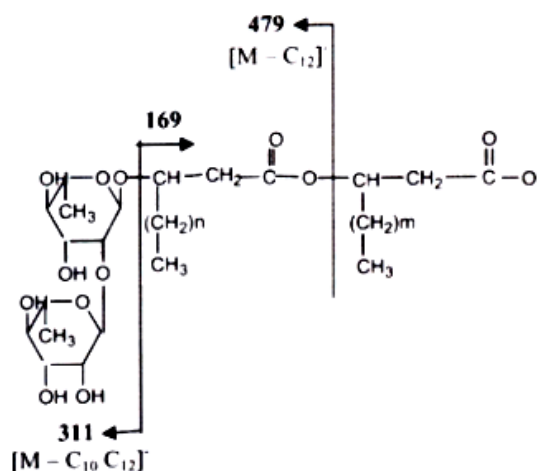


Figure 4. Principal fragmentations of dirhamnolipids produced by *Pseudomonas aeruginosa* LBI. The base fragmentations were: m/z 479, m/z 311 and m/z 169 for dirhamnolipids; m/z 333 and m/z 163 for monorhamnolipids (Benincasa *et al.*, 2004).

al., 2002). Biosurfactant production increased after nitrogen depletion and the maximal rhamnolipids concentration was 15.9 g/l. High performance liquid chromatography mass spectrometry (HPLC-MS) analysis revealed that the purified culture supernatant was 6 RL homologues (Figure 4). RL_{LBI} produced stable emulsions with hydrocarbons (crude oil, kerosene, toluene, n -alkanes (C_{12} - C_{14}) and mineral oil) and vegetable oils (linseed oil, almond oil). In addition, this product exhibited a good antimicrobial activity against bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, *Proteus vulgaris*, *Streptococcus faecalis* and *Pseudomonas aeruginosa*) and phytopathogenic fungal species (*Penicillium*, *Alternaria*, *Gliocadium virens* and *Chaetonium globosum*) (Benincasa *et al.*, 2004).

Molasses: Molasses is a co-product of sugar production, both from sugar cane as well as from sugar beet. It is defined as the runoff syrup from the final stage of crystallization, in which further crystallization of sugar is uneconomical. Molasses generally consists of 48-56% total sugar (mainly sucrose), 9-12% non-sugar organic matter, 2-4% protein ($N \times 6.25$), 1.5-5% potassium, 0.4-0.8% calcium, 0.06% magnesium, 0.6-2.0% phosphorus, 1.0-3.0 mg/kg biotin, 15-55 mg/kg pantothenic acid, 2500-6000 mg/kg inositol and 1.8 mg/kg thiamine (Makkar and Cameotra, 1997). Molasses has been used as the major raw material for production of pullulan (Lazaridou *et al.*, 2002), xanthan gum (Kalogiannis *et al.*, 2003), baker' yeast (Skountzou *et al.*, 2003), citric acid (Ikram-ul *et*

al., 2004) as well as fructo-oligosaccharides (Shin *et al.*, 2004).

Ghurye and Vipulanandan (1994) used activated sludge from wastewater treatment as a source of microorganisms for biosurfactant production. A molasses concentration of 20 g/l was used as a carbon source. The production of biosurfactant appeared to be associated with growth since the critical micelle dilution (CMD) and emulsification capacity increased with increasing biomass. Biosurfactant might consist of proteins or peptides moieties because pronase lowered the emulsification capacity of the cell-free broth.

Bacillus subtilis MTCC 2423 and *Bacillus subtilis* MTCC 1427 were cultivated using molasses (2% total sugar) as a carbon source and incubated at thermophilic condition (45°C). Maximal biosurfactant production as evidenced by surface tension lowering was achieved from both strains in the late stationary phase. However, strain MTCC 2423 produced greater biosurfactant content than strain MTCC 1427. As a result of biosurfactant accumulation, the surface tension of the medium was lowered to 29 and 31 dynes/cm by MTCC 2423 and MTCC 1427, respectively. Additionally, oil recovery from sand pack column was 34% for MTCC 1427 and 38.46% for MTCC 2423, indicating the potential use of these biosurfactants in the enhanced oil recovery (Makkar and Cameotra, 1997).

Patel and Desai (1997) used the molasses and cornsteep liquor as the primary carbon and nitrogen source to produce rhamnolipid biosurfactant from *Pseudomonas aeruginosa* GS3. The biosurfactant production (quantified by measuring the interfacial tension and expressing rhamnolipids in term of rhamnose) reached the maximum when 7% (v/v) of molasses and 0.5% (v/v) of cornsteep liquor were used. Maximal surfactant production occurred after 96h of incubation, when cells reached the stationary phase of growth. A rhamnose concentration of 0.25 g/l and a reduction of interfacial tension between surfactant and crude oil of up to 0.47 mN/m were obtained.

Whey: Whey is a liquid by-product of cheese or tofu production containing the water soluble

components. The organic matter in whey causes a high biological oxygen demand (BOD) in the range of 40-60 g/l (Ghaly and Kamal, 2004) and high chemical oxygen demand (COD) in the range of 40-70 g/l (Lee *et al.*, 2003). Thus it must be treated before disposal into waterways and sewerage systems. To date, the disposal of whey is still an important environmental problem. An alternative to chemical treatment and disposal is the use of whey as a substrate for the growth of microorganisms such as *Ganoderma lucidum* (Lee *et al.*, 2003), *Acetobacter xylinus* (Battad-Bernardo *et al.*, 2004) and *Kluyveromyces fragilis* (Ghaly and Kamal, 2004).

Dubey and Juwarkar (2001) cultivated *Pseudomonas aeruginosa* BS2 on whey waste for biosurfactant production. Within 48 h of incubation the yield of biosurfactant obtained was 0.92 g/l. Strain BS2 produced a crystalline biosurfactant (Figure 5) as the secondary metabolites and its maximal production occurred after the onset of nitrogen-limiting conditions. The isolated biosurfactant possessed the potent surface-active properties, as it effectively reduced the surface tension of water from 72 to 27 mN/m and formed 100% stable emulsion of a variety of water-insoluble compounds. After recovering biosurfactant from the fermented waste, COD, total acids, nitrogen and phosphate levels in the whey were significantly reduced by 87, 90, 92 and 92%, respectively.

Starch-rich wastes: The processing of agro-industrial raw materials such as cassava or potato produces the large amount of waste, whose accumulation leads to environmental pollution. Due to the high amounts of starch or reducing sugar, those wastes has been recognized as a suitable feedstock for industrial fermentations such as production of pullulan (Barnett *et al.*, 1999) and volatile compounds (Christen *et al.*, 2000).

Potato substrates were evaluated as a carbon source for surfactant production by *Bacillus subtilis* ATCC 21332. Surface tensions dropped from 71.3 mN/m to 28.3 mN/m and 27.5 mN/m when potato medium and mineral salt medium were used, respectively. The critical micelle con-

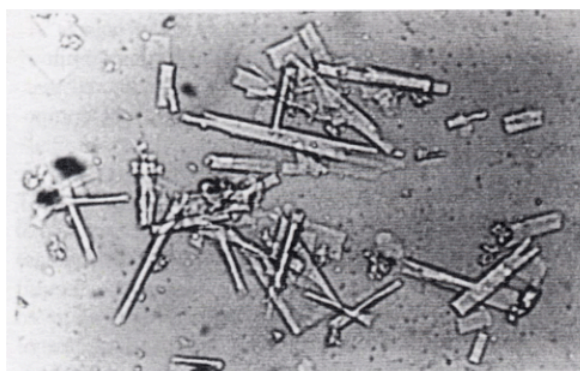


Figure 5. Microscopic observation of crystalline form of biosurfactant from *Pseudomonas aeruginosa* BS2 (Dubey and Juwarkar, 2001).

centration (CMC) of 0.10 g/l was obtained from a methylene chloride extract of the potato solid medium (Fox and Bala, 2000). Furthermore, high-solids (HS) and low-solids (LS) potato process effluents were used as substrates for surfactin (Figure 6) production by *B. subtilis* ATCC 21332 (Thompson *et al.*, 2000). Surfactin production from LS potato effluent gave the greater yield (0.39 g/l) than that from HS potato effluent (0.097 g/l). The fourier transform infrared spectroscopy (FTIR) of commercial surfactin and the LS precipitate revealed that the biosurfactant produced was surfactin (Figure 7). In addition, Nitschke and Pastore (2004) used a cassava flour-processing effluent as a substrate for surfactant production by *Bacillus subtilis* LB5a and *Bacillus subtilis* ATCC 21332. *B. Subtilis* ATCC 21332 reduced the surface tension of the medium from 49.5 mN/m to 25.9 mN/m and produced a crude biosurfactant at a concentration of 2.2 g/l. For *B. subtilis* LB5, the

surface tension of the medium was reduced to 26.6 mN/m, giving a crude biosurfactant concentration of 3.0 g/l. The FTIR spectra of commercial surfactin and the semipurified surfactant produced by strain LB5a indicated that the product obtained was surfactin-like surfactant (lipopeptide).

Conclusion

The main factor limiting commercialization of biosurfactants is associated with non-economical large-scale production. To overcome the obstacle and to compete with synthetic surfactants, inexpensive substrate and effective microorganism has to be intensively developed for biosurfactant production. Agro-industrial wastes are considered as the promising substrate for biosurfactant production and can alleviate many processing industrial waste management problems.

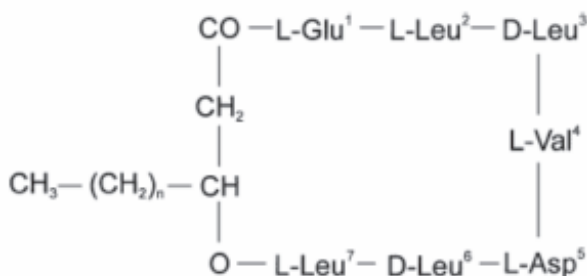


Figure 6. Primary structure of surfactin (n = 9-11) (Morikawa *et al.*, 1992).

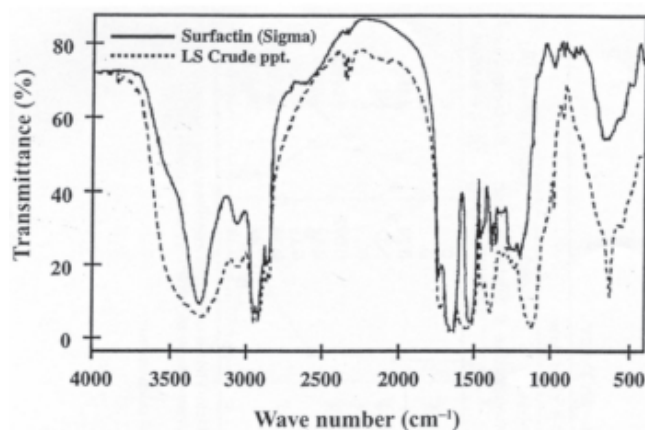


Figure 7. Transmission FTIR spectra of commercial surfactin and the surfactant produced by *B. subtilis* ATCC 21332 using low-solid potato process effluent. (Thompson *et al.*, 2000).

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